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Substrate specificity of DNA polymerases. I. Enzyme-catalysed incorporation of 5-(1-alkenyl)-2'-deoxyuridines into DNA

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ABSTRACT

A series of (E)-5-(1-alkenyl)-dUTPs as well as 5-vinyl- and (Z)-5-(1-propenyl)-dUTP have been synthesized to study steric requirements in DNA polymerase reactions. Experiments were carried out in E. coli DNA polymerase I Klenow fragment enzyme system. Substrates were characterized by K_M and v_{max}-values, initial incorporation rates as well as by total extent of incorporation of the analogues into poly(dA-dT) as a template-primer.

Incorporation of the analogues could be best correlated with v_{max}-values as well as the very similar initial incorporation rate values. Reactivity (v_{max}/K_M) showed no correlation with the extent of incorporation. 5-Vinyl-dUTP proved to be as good a substrate of the enzyme as dTTP, whereas (E)-5-(1-heptenyl)- and (E)-5-(1-octenyl)-dUTPs were very poor substrates, their incorporation was strongly limited and they also proved to be very efficient inhibitors of DNA replication, as shown by K_i-values.

Substrate specificity of the Klenow enzyme can be explained by the steric hindrance of C-5 substituent, by the "orientational steric substituent effect" concept.

INTRODUCTION

Template-determined incorporation of a given natural nucleotide unit into a DNA in an *in vitro* replication system has been generally accepted as a classical example of the highly specific recognition processes between biopolymers and small molecules. Explanation of the phenomenon by base-pairing is so clear and well-known that it may be called "trivial" or "base-pairing-determined" substrate specificity in DNA polymerase catalyzed reactions.

Investigation of several dozens of analogues of nucleoside triphosphates in the above reaction at different laboratories showed that the incorporation into a DNA varies by orders of

magnitude even if the structural elements of base-pairing are ensured. This latter "secondary" or "non-base-pairing-determined" (not only base-pairing-determined) substrate specificity can be explained by the "orientational steric substituent effect" and "compressional steric substituent effect" concepts worked out on other biopolymer transformations (1,2,3) including cross-linking alkylation of DNA (4,5). In this paper the DNA polymerase-catalyzed reactions of 5-(1-alkenyl)-dUTPs are presented. Discussion of the results is extended also on the substrate specificity observed earlier with 5-alkyl-dUTPs.

EXPERIMENTAL

Materials

Klenow fragment enzyme of E. coli MRE 600 DNA polymerase I (7000 units/mg protein), poly(dA-dT), dATP, dTTP, dGTP and dCTP were purchased from Boehringer-Mannheim GmbH. Calf thymus DNA was from Miles-Seravac and was activated according to Aposhian and Kornberg (6). [^{3}H]dATP (17 Ci/mmol) was from New England Nuclear. [^{3}H]dTTP (21.2 mCi/mmol) was prepared from [^{3}H]thymidine according to (10).

Synthesis of (E)-5-(1-alkenyl)-dUTPs

The modified nucleosides were prepared by known procedures: 5-vinyl-dUrd according to (7), (E)-5-(1-propenyl)-dUrd to (8), (Z)-5-(1-propenyl)-dUrd, (E)-5-(1-butenyl)-dUrd, (E)-5-(1-pentenyl)-dUrd and (E)-5-(1-hexenyl)-dUrd accoring to (9). (E)-5-(1-Heptenyl)-2'-deoxyuridine and (E)-5-(1-octenyl)-2'-dezoxyuridine were prepared according to the procedure in (9). Physical characteristics are as follows.

(E)-5-(1-heptenyl)-dUrd: mp. 115-117 ^{o}C, R_f value was 0,57 in EtOAc-MeOH (95:5), $UV_{\lambda max}$ (MeOH) 241,298 nm, ^{1}H-NMR peaks, δ (at 100 MHz in DMSO-d_6 with Me_4Si internal standard) 9.30(bs,3N-H); 7.75(s,6C-H); 6.44(dt,vinylic proton); 6.28(t, H-1'); 6.12(d,vinylic proton,J=16 Hz); 4.50(m,H-3'); 4.00(m, H-4'); 3.80(m,H-5'); 2.2-2.4(m,H-2'); 2.15(m,allylic proton), 1.15-1.60(side-chain CH_2); 0.91(side-chain CH_3).

(E)-5-(1-octenyl)-dUrd: mp. 134-136 ^{o}C, R_f value 0.59, $UV_{\lambda max}$ 241,299 nm and ^{1}H-NMR peaks, δ : 10,85(bs,3N-H), 7.39(s, 6C-H); 6.42(dt,vinylic proton); 6.25(t,H-1'); 6.08(d,vinylic

proton,J=15.5 Hz); 4.35(m,H-3'); 3.88(m,H-4'); 3.70(m,H-5'); 1.9-2.4(m,H-2'); 2.1(m,allylic proton); 1.1-1.6(side-chain $C\underline{H}_2$); 0.88(side-chain $C\underline{H}_3$).

Synthesis of the corresponding 5'-triphosphates was based on the procedure described by Ludwig (10). (Synthesis of 5-vinyl-dUTP will be published elsewhere.) A known amount of nucleoside (0.3 mmole) was stirred in dry trimethyl phosphate (0.75 ml) at 0°C. Phosphorous oxychloride (60 µl, 2.2 eq) was added, the mixture was stirred at 0-4 °C. After maximal formation (70-90 %) of the intermediate nucleoside phosphorodichloridate was observed (12-24 hrs, strongly dependent on the analogue), a mixture of 0.5 M bis-tri-n-butylammonium pyrophosphate in anhydrous DMF (3 ml, 5 eq.) and Bu_3N (0.3 ml) was quickly added to the reaction mixture under vigorous stirring at 0 °C. After 1 min. 0.2 M aqueous $Et_3N.H_2CO_3$, pH 7.5 (30 ml) was poured into the solution. After evaporation the residue was separated by column chromatography on DEAE-Cellulose (Whatman, DE 32) using a linear gradient of triethylammonium bicarbonate between 0 and 400 mM (800-800 ml) at 4 °C. After purification the 5'-triphosphate was converted into tetrasodium salt.

On this scale, isolated yields of analogue triphosphate ranged from 40 to 65 % of theoretical from the nucleoside.

In all cases, the observed UV absorption profile was unchanged from parent nucleoside. Structure of modified nucleoside 5'-triphosphate was confirmed by NMR data. The corresponding nucleotides have coupling constants for the vinylic protons of J=16-18 Hz and have been assigned the E stereochemistry in the side-chain.

The (Z)-5-(1-propenyl)-dUTP has a coupling constant for the olefinic protons of J=11.5 Hz.

Purity of 5'-triphosphates, that was at least 90 %, was checked by TLC scanning (Shimadzu TLC Scanner, CS-920). R_f values on silica plate (Kieselgel 60 F_{254}, Merc) are as follows in n-propanol-NH_3-H_2O (11:7:2): dTTP 0.18, 5-vinyl-dUTP 0.16, (E)-propenyl- 0.21, (Z)-propenyl- 0.19, butenyl- 0.24, pentenyl- 0.27, hexenyl- 0.28, heptenyl- 0.31 and (E)-5-(1-octenyl)-dUTP 0.33.

Methods

Six types of DNA polymerase assay were carried out: a./ determination of initial rate (30 min.) and b./ total extent (24 hours) of incorporation of 5-substituted dUTPs into poly(dA-dT) as a template-primer; c./ measurement of the ability of a few modified nucleotides to affect dATP + dTTP polymerization on poly(dA-dT); d./ determination of the substrate selectivity of Klenow DNA polymerase in the presence of activated calf thymus DNA as a template-primer; e./ determination of K_M-values for the modified dUTPs; and f./ K_i-values for two selected dUTP analogues.

Reaction mix for Klenow polymerase consisted of 60 mM potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. dTTP or 5-(1-alkenyl)-dUTP was present at 200 μM concentration, [^{3}H]dATP (4.7 mCi/mmol) at 100 μM and poly(dA-dT) was 100 μM(P) in assays a and b. In assay d dGTP and dCTP were also present in 100-100 μM and activated calf thymus DNA in 150 μg/ml concentrations. In inhibition experiments of c effect of different concentrations of a few selected 5-(1-alkenyl)-dUTPs on replication rate (30 min) of 100 μM dTTP and 100 μM of [^{3}H]dATP in the presence of 100 μM(P) of poly(dA-dT) was assayed.

For the determination of K_M-values (e) with poly(dA-dT) (50 μM/P/) 100 μM [^{3}H]dATP (28.3 mCi/mmol) and 0.01-50 μM dUTP analogues (8 different concentrations) were applied. K_M-values were calculated from S/V-S plotting of the data using a Sinclair ZX Spectrum microcomputer with a program for linear regression analysis. K_i-values were measured using 50 μM(P) poly(dA-dT), 100 μM dATP, 5 different concentrations of [^{3}H]dTTP (21.2 mCi/mmol) in the range of 2.5-50 μM and 0.05-2 μM inhibitor (2-3 concentrations). K_i-values were determined from both S/V-S and 1/V-1/S plots using the above program for least square analysis.

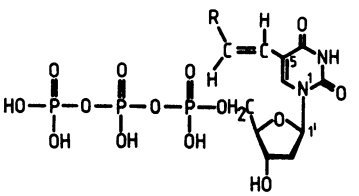
Final volume in experiments a, c, d, e and f was 50 μl and 0.5 μg of Klenow enzyme was added to start synthesis in the case of a, c and d, whereas 0.2 μg with e and f assays. After 30 minutes of incubation at 37 °C 25 μl samples were taken and worked up as described below. In experiments b final volume was 100 μl and 1 μg of polymerase was added, and 10 μl samples were taken at 0.5, 1, 2, 5 and 24 hours of incubation to follow synthesis.

10 and 25 µl samples taken from reaction mixtures were spotted onto GF/C (Whatman) filters of 2.4 cm diameter. Filters were collected in 5 % trichloroacetic acid containing 1 % of sodium pyrophosphate, washed two times with 5 % trichloroacetic acid solution, two times with ethanol, then with diethylether and finally air-dried. Radioactivity of acid-insoluble polymeric product was counted in LKB 1217 Rackbeta spectrometer. Data given in the Table and Figures are mean values of 2 to 6 experiments in diplicate.

RESULTS

Substrate specificity of DNA polymerase with poly(dA-dT)

First insight into the stereochemical course of substrate selection by the Klenow fragment DNA polymerase enzyme with 5-(1-alkenyl)-dUTPs was obtained by measuring initial incorpora-



(E)-5-(1-alkenyl)-dUTP

tion rate of [^{3}H]dAMP into poly(dA-dT) in the presence of each 5-(1-alkenyl)-dUTP and dTTP. Figure 1 shows the relative ability of the modified nucleotides to substitute for dTTP in this system, and Table 1 collects data.

5-Vinyl-dUTP was as good substrate of the enzyme as dTTP. Substitution of the (E)-2 proton of 5-vinyl group by alkyl chains decreased the ability of nucleotides to substitute for dTTP in this synthesis reaction. The decrease was not linear with alkyl chain length. Beyond experimental error there was a drop in rate with (E)-5-(1-propenyl)-dUTP. Relative rate of the pentenyl- and hexenyl-analogues was around 50 %. A sharp drop in rate came after the hexenyl analogue: the heptenyl- and octenyl-dUTPs were hardly any substrates.

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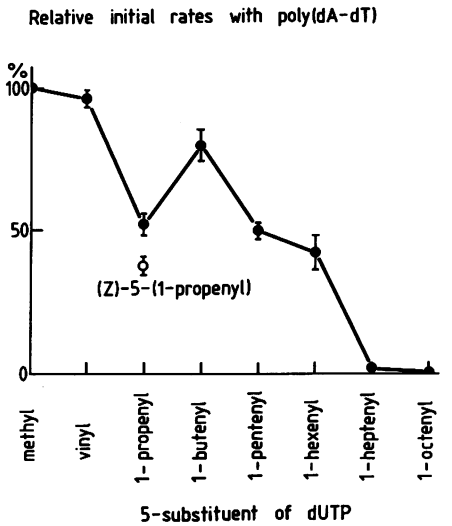

Figure 1. Substrate specificity of Klenow DNA polymerase for 5-(1-alkenyl)-dUTPs in the presence of poly(dA-dT) (data were taken from Table 1)

enzyme came from the 24 hour kinetic data: these give the total extent of incorporation of the analogues, i.e. extent of copolymerization with [^{3}H]dAMP starting from unlabeled poly-(dA-dT). With dTTP as well as with 5-vinyl-dUTP saturating values (more than 95 % of substrate use) have already been reached during 5 hours of incubation. With the exception of the propenyl and butenyl analogues only a slight increase in incorporation was observed after 5 hours with the other analogues. Data are shown in Table 1.

5-Vinyl-dUTP proved to be as good substrate as dTTP. Except for propenyl- and butenyl-dUTPs every other analogue retained at 24 hours the initially measured relative value. Both the propenyl and butenyl derivatives reached a higher final relative incorporation than their relative initial rate.

Relative v_{max}-values for the analogues, v_{max}-s compared to the v_{max} for dTTP of 0.276 nmol/25 µl, 30 min, are in good agreement with the relative initial incorporation rate values measured at 30 min (Table 1). (Latter values were obtained with 200 µM concentration of dTTP analogues, corresponding to

Table 1. Kinetic data for the incorporation of 5-(1-alkenyl)-dUTPs into poly(dA-dT) and calf thymus DNA by Klenow DNA polymerase enzyme

Substrate	poly(dA-dT) template-primer						Activated calf thymus DNA
	relative incorporation (%)* 30 min rate	relative incorporation (%)* extent at 24 h	K_M (μM)	relative V_{max} (%)***	rel.V_{max} / K_M	K_i (μM)	incorporation rate at 30 min (%)**
dTTP	100	100	4.1 ± 0.5	100	24.4		100
5-vinyl-dUTP	96.0 ± 2.6	100 ± 2.1	5.4 ± 0.2	105 ± 4	19.4		88.7 ± 1.5
(E)-5-(1-propenyl)-dUTP	52.1 ± 4	80 ± 5	1.0 ± 0.2	52.0 ± 7	52.0		74.7 ± 4.8
(E)-5-(1-butenyl)-dUTP	80.0 ± 6	88.5 ± 3.5	4.1 ± 0.4	78.0 ± 5	19.0		67.6 ± 2.1
(E)-5-(1-pentenyl)-dUTP	50.0 ± 2	45.1 ± 4.2	3.5 ± 1.1	62.5 ± 1.5	17.9		36.8 ± 5
(E)-5-(1-hexenyl)-dUTP	42.6 ± 6	40.5 ± 3.5	4.2 ± 0.5	58.0 ± 4	13.8		25.6 ± 4.3
(E)-5-(1-heptenyl)-dUTP	1.5 ± 0.6	1.3 ± 0.5	0.23 ± 0.05	1.2 ± 0.3	5.2	0.6 ± 0.05	18.5 ± 2.8
(E)-5-(1-octenyl)-dUTP	0.2 ± 0.1	0.4 ± 0.2	0.03 ± 0.01	0.3 ± 0.1	10.0	0.35 ± 0.12	12.3 ± 0.8
(Z)-5-(1-propenyl)-dUTP	37.5 ± 3	34.0 ± 2.5	19.6 ± 1.1	25.0 ± 2	1.3		12.1 ± 0.3

* Relative rates were calculated by comparing rates in the presence of modified nucleotides to that of the dTTP reaction. With poly(dA-dT) the latter was 7008 dpm in the 25 μl aliquot, taken at 30 minutes of incubation, corresponding to 0.667 nmol of [^{3}H]dAMP (4.7 mCi/mmol) incorporation/25 μl.30 min. (26.4 % of substrate consumption). From this value 0.004 nmol, value measured in the same volume and 30 min. in the presence of only one substrate ([^{3}H]dATP), was substracted and net value was taken as 100 %. The same values with poly(dA-dT) in the 10 μl aliquot at 24 hours were: 10435 dpm (0.994 nmol, 98.4 %) and 0.003 nmol was substracted.

** With activated calf thymus DNA values in the presence of dTTP in 25 μl aliquot at 30 min. of incubation were: 5273 dpm (0.502 nmol, 19.9 %), substracted value of the 3 substrate "limited reaction" was 0.016 nmoles.

*** Relative V_{max}-values were calculated by comparing V_{max}-values for dUTP analogues to that of the dTTP that corresponded to 0.276 nmol/30 min, 25 μl (17340 dpm from [^{3}H]dAMP (28.3 mCi/mmol) incorporation/30 min, 25 μl; 11 % substrate consumption) after substraction of 4.0 pmol (250 dpm/30 min, 25 μl) obtained in the presence of only [^{3}H]dATP.

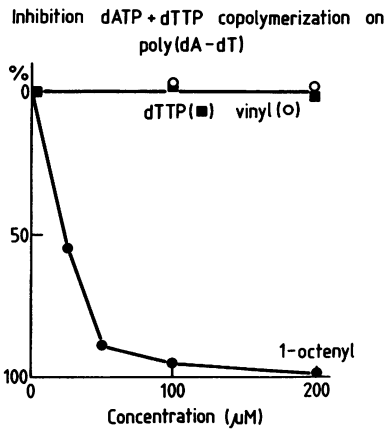

Figure 2. Inhibition of replication of poly(dA-dT) by 5-(1-alkenyl)-dUTPs
The 0 % inhibition (i.e. 100 % synthesis) of the 100 μM dTTP and 100 μM [^{3}H]dATP reaction in the presence of 100 μM(P) of poly(dA-dT) corresponds to 5934 dpm (0.565 nmoles, 22.4 % substrate use) measured in the 25 μl aliquot taken at 30 min. of incubation at 37 °C.

at least 30 times of K_M, and both measurements were carried out at the linear part of the polymerization process at low conversion: around 10 % and 25 %, respectively, of substrate conversion with dTTP, with the use of 0.2 and 0.5 μg of Klenow enzyme in the K_M and the 30 min rate reactions, respectively.) The differences observed were with the pentenyl and hexenyl derivatives where rel. v_{max}-values were higher than rel. initial rates, and with Z-propenyl-dUTP where rel. v_{max} was lower.

K_M-values for the 5-vinyl-, butenyl-, pentenyl- and hexenyl-dUTPs were very similar to that of the dTTP. K_M-s for the stereoisomers of 5-propenyl-dUTP deviated from K_M for dTTP in both directions reflecting a special binding feature of the propenyl side chain with Klenow enzyme complexed with poly(dA-dT) template. The greatest differences in K_M were observed for the (E)-5-(1-heptenyl)- and (E)-5-(1-octenyl)-dUTPs, values were lower by one and two orders of magnitude, respectively, than that of the dTTP.

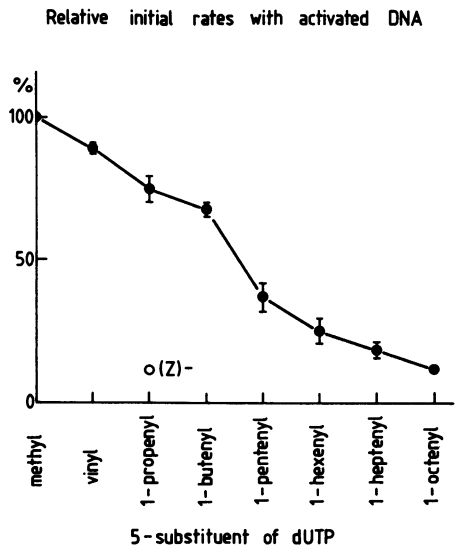

Figure 3. Substrate specificity in the presence of activated calf thymus DNA (data are from Table 1)

Effect on replication of dTTP + dATP

The ability of the very good substrate 5-vinyl-dUTP and the poorest substrate (E)-5-(1-octenyl)-dUTP, respectively, to affect copolymerization of dTTP with [^{3}H]dATP on poly(dA-dT) primer-template is shown on Figure 2. (Effect of increasing concentration of dTTP was also tested: no increase in rate was observed, v_{max}-value has already been reached with 100 μM of dTTP.)

5-Vinyl-dUTP did not change dATP + dTTP replication: it acted as if it were dTTP. Adding the 5-octenyl-dUTP rate of copolymerization of natural substrates decreased sharply. Since very high dTTP concentration was applied this inhibition should be pronounced. Indeed, K_i-value for 5-octenyl-dUTP was around 1/10th of the K_M-value for dTTP. (It is important to note that it was a mixed type of inhibition.) In this way, 5-octenyl-dUTP is a very effective inhibitor of DNA replication catalyzed by isolated DNA polymerase enzyme.

Substrate selection with calf thymus DNA

Figure 3 and Table 1 shows the data for relative initial rate of incorporation of [^{3}H]dAMP into activated calf thymus DNA in the presence of 5-(1-alkenyl)-dUTPs, catalyzed by Klenow DNA polymerase enzyme.

The same DNA polymerase enzyme showed a different substrate selection pattern with this four-base natural template-primer. Relative rates decreased almost linearly with increasing number of carbon atoms in the side chain of 5-(1-alkenyl)-dUTP, and even heptenyl- and octenyl-dUTPs were substrates.

DISCUSSION

The explanation of substrate specificity, including stereospecificity, in enzyme-catalyzed reactions by "orientational steric substituent effect" (OSSE) conception is based on the fact that kinetics of decomposition of an ES (enzyme-substrate) complex or transformation of ES'-compound into the direction of the product is determined by the steric requirement of a given substituent of the substrate oriented towards the reac-

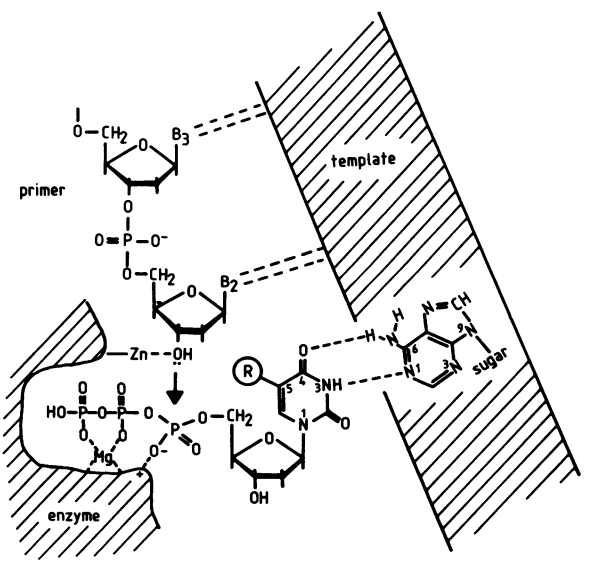

Figure 4. Schematic representation of enzymatic DNA synthesis

tion centers of the reactants. This steric arrangement is ensured by two-locus interactions between macromolecule and substrate. In the DNA polymerase /substrate/template/primer complex, represented schematically in Fig. 4., the two fixed molecular moieties of dTTP or the (E)-5-(n-1-alkenyl)-dUTP are the 3-NH and 4-CO groups of the pyrimidine ring bound to template (base-pairing) on one hand and the triphosphate chelate fraction of the molecule bound to the enzyme on the other (11). In this way, the position of the 5-substituent is determined. In the case of dTTP-Mg^{2+} chelate the glycosidic torsional angle is, as recently determined, 40 ± 10 ^{o} (anti) when the chelate is bound to the Klenow fragment enzyme with or without a template (12,13). Thus the 5-(1-alkenyl) substituent oriented toward the 5'-phosphate group of the dUTP, depending on its steric requirement, may hinder the nucleophilic attack of the Zn-activated 3'-OH end-group of the primer terminus in the reaction.

Measure of the steric hindrance depends on the length and shape of the substituent. The C_1, C_2 and C_3-atoms of the 5-(1-alkenyl) group must have a zig-zag form, either as drawn on the structural formula for 5-alkenyl-dUTP here or the C_2-atom pointing "upwards" as was determined in solid state for (E)-5-(2-bromovinyl)-dU (14). C_1 and C_2 atoms lie in the plane of the pyrimidine ring as a consequence of conjugation. Either way, the whole group is oriented toward the reaction center. Accepting this, a moderate continous decrease in v_{max}-values can be expected for the analogues with increasing length in side chain based on the "OSSE" concept: beyond C_2-atom of the side chain a twisted conformation, owing to the rotation around C_2-C_3, etc., can be assumed. In this was, however, after a given chain length, determined by the steric characteristics of the active center of the enzyme complexed with the template-primer and the bulkiness of side chain in its zig-zag form, a sharp drop in v_{max} can also be foreseen.

Indeed, experimental results listed in Table 1 show that there was a decrease in relative v_{max} (and relative initial incorporation rate) values, 100 to 58 (100 to 43) per cent, for the methyl to hexenyl-dUTPs and a strong decrease with the hep-

tenyl and octenyl derivatives. The little lower than expected v_{max} for the (E)-5-(1-propenyl)-dUTP is unexplained and is template-dependent (see later).

Both stereoisomers of the 5-propenyl-dUTP showed K_M-values, which values are the next kinetic characteristics of the system to be discussed, different from that of the average around 5-propenyl-dUTP: K_M-values were very similar for dTTP and the analogues up to the hexenyl derivative (Table 1). K_M for the E-isomer of 5-propenyl-dUTP was about $1/4^{th}$ of that of dTTP, whereas for the Z-isomer it was about 5 times higher than for dTTP and 20 times higher than for the E-isomer. There should be a special steric arrangement in the enzyme-DNA complex that prefers the binding of the E-isomer while is very disadvantageous for the Z-isomer. The low v_{max} for the Z-isomer may originate from the weak binding of the compound.

Surprisingly, dUTP analogues that exhibited much lower rate of incorporation than Z-propenyl-dUTP, the 5-heptenyl- and 5-octenyl-dUTPs, possessed one and two orders of magnitude, respectively, stronger binding affinity to the Klenow enzyme complexed with poly(dA-dT), as are shown by K_M-values.

The low K_M-s for the heptenyl- and octenyl-derivatives, compared to that of the dTTP, suggest also a strong inhibition of replication. Figure 2 shows a definite inhibition of dTTP + + dATP polymerization on poly(dA-dT) template in comparison with 5-vinyl-dUTP which is as good substrate as dTTP, and did not influence replication under conditions applied. The inhibition by the long-chain analogues was quantified by measuring K_i-values (Table 1). Type of inhibition was not a competitive type but of mixed type (curves not shown).

Based on the relative V_{max}/K_M-values (Table 1) efficiency of 5-octenyl-dUTP as a substrate should be very similar to that of the 5-hexenyl-dUTP or even dTTP. According to the total incorporation values determined at 24 h of incubation at 37 ^{o}C, however, incorporation of 5-octenyl- and also 5-heptenyl-dUTPs was strongly limited, and did not reach 2 % of that of dTTP (Table 1). Therefore, in addition to K_M and v_{max} or K_i-values, by which most enzyme-substrate systems can be characterized, for the complete description of substrate properties of a nucleotide in a template-primer-dependent DNA polymerase system deter-

mination of a further parameter, the total extent of incorporation of the analogue may be required. Based on the present results, however, relative v_{max} or initial incorporation rate values are very well correlated with total extent of incorporation.

Since total incorporation values are very much different for the analogues a further steric factor should be taken into account. This may come from the analogue having incorporated into the growing primer chain. Depending on the steric requirement of the side chain of the incorporated analogue a conformational modification of the growing primer chain occurs. After a certain degree of deformation the primer terminus can no more function, it is not at the right place. The 5-heptenyl- and 5-octenyl-dUTPs produced the most pronounced examples: their incorporation is limited only to a few analogues plus dAMP per template-primer molecule. In this way, helical parameters of the template-primer DNA around the active center of the enzyme affect the extent of substrate usage.

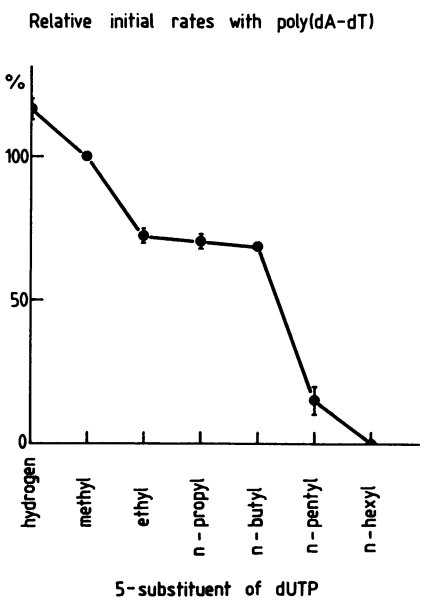

Figure 5. Substrate specificity of the enzyme tested with 5-alkyl-dUTPs in the presence of poly(dA-dT) (16).

Figure 3 and Table 1 with the data, presents one further example on the importance of template-primer structure in substrate analogue incorporation by DNA polymerase enzyme. Activated calf thymus DNA was used and relative initial incorporation rates were determined. Substrate specificity with this four-base DNA was not so high as observed with poly(dA-dT), and even (E)-5-(1-octenyl)-dUTP was a substrate of the Klenow enzyme, approximately only one order of magnitude weaker than dTTP.

Comparison of the poly(dA-dT) and thymus DNA systems shows the importance of the structure of template-primer not only in the "base-pair-determined" but also in the "secondary" substrate specificity of DNA polymerase-catalyzed reactions. Base sequence or base sequence-dependent local polynucleotide conformation or both are supposed to affect the extent of substrate analogue incorporation into the growing primer chain as well as the kinetics of incorporation. Role of the structure of template-primer in binding properties of the analogues (K_M, K_i) is under investigation.

In addition to the length in a series of 5-substituted dUTP analogues chemical composition of the side chain affects its incorporation through hydrophobicity and steric arrangement. This is, probably, the explanation that a very long chain analogue of dTTP, 5-biotinyl-dUTP can well substitute for dTTP (15).

Analysis of the results obtained in earlier experiments with 5-alkyl-dUTPs (16) extends further the explanation of "non-base-pairing-determined" substrate specificity in DNA polymerase catalysed reactions. One example on structure-incorporation relationship is presented on Figure 5. In this case also poly(dA-dT) and E. coli DNA polymerase I enzyme were used (16). Results are very similar to those observed in the present series of (E)-5-alkenyl-dUTPs. The main difference is that the drop in relative initial incorporation rate was observed at 5-pentyl-dUTP in the series of 5-alkyl-dUTPs and at the seven-carbon-atom analogue in the case of 5-alkenyl-dUTPs. The absence of double bond in the former series allows rotation around C_1-C_2 atoms of the side chain and thus the formation of a twisted conformation. Consequently, higher steric effect may occur at a shorter-lenght-substituent. The "OSSE" was found to be valid in DNA polymerase-

catalyzed reactions of 5-alkyl-dUTPs, employing also other enzymes and templates too (17).

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